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Research Report

Cholesterol biosynthesis and the pro-apoptotic effects of the p75 nerve growth factor receptor in PC12 pheochromocytoma cells

Chaohua Yan[a], Zeljka Korade Mirnics[a], Carmel F. Portugal[a], Ye Liang[a], Karen D. Nylander[a], Marcelo Rudzinski[b], Clara Zaccaro[b], H. Uri Saragovi[c], Nina Felice Schor[a,*]

[a]*Division of Child Neurology, The Pediatric Center for Neuroscience, Children's Hospital of Pittsburgh, 3705 Fifth Avenue, Pittsburgh, PA 15213, USA*
[b]*McGill University Cancer Center, Montreal, Quebec, Canada*
[c]*Lady Davis Institute-Jewish General Hospital, Montreal, Quebec, Canada*

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Abstract

Neocarzinostatin (NCS), an enediyne antimitotic agent, induces cell death in both p75NTR neurotrophin receptor (NTR)-positive and p75NTR-negative PC12 cells in a concentration-dependent fashion. However, p75NTR-positive cells demonstrate a higher susceptibility to NCS-induced cell damage. Furthermore, treatment of p75NTR-positive cells with the p75NTR-specific ligand, MC192, resulted in apoptosis, while treatment of these cells with the TrkA-specific ligand, NGF–mAbNGF30, protected them from NCS-induced death, implying that both the naked and liganded p75NTR receptors have a pro-apoptotic effect on PC12 cells. Microarray studies aimed at examining differential gene expression between p75NTR-positive and p75NTR-negative cells suggested that enzymes of the cholesterol biosynthetic pathway are differentially expressed. We therefore tested the hypothesis that altered cholesterol biosynthesis contributes directly to the pro-apoptotic effects of p75NTR in this PC12 cell-NCS model. Subsequent Northern blotting studies confirmed that the expression of p75NTR is associated with the upregulation of cholesterol biosynthetic enzymes including 3-hydroxy-3-methylglutaryl CoA reductase (HMG CoA reductase), farnesyl-diphosphate synthase, and 7-dehydro-cholesterol reductase. Mevastatin, an HMG CoA reductase inhibitor, converts the apoptosis susceptibility of p75NTR-positive cells to that of p75NTR-negative cells. It does so at concentrations that do not themselves alter cell survival. These studies provide evidence that the pro-apoptotic effects of p75NTR in PC12 cells are related to the upregulation of cholesterol biosynthetic enzymes and consequent increased cholesterol biosynthesis.

Theme: Development and regeneration
Topic: Neurotrophic factors: receptors and cellular mechanisms

Keywords: p75 neurotrophin receptor; Nerve growth factor; Apoptosis; Cholesterol

Nerve growth factor (NGF), the best characterized member of the neurotrophin family, plays an essential role in the growth, survival, and differentiation of neurons in both the peripheral and central nervous systems [32]. The varied activities of NGF are mediated by the TrkA/p75 neurotrophin receptor (NTR) complex tyrosine kinase receptor, a high-affinity receptor with a K_d of 10^{-11} M, and p75NTR, a low-affinity receptor with a Kd of 10^{-9} M and a member of the tumor necrosis factor death receptor family [1,15,35,53]. TrkA has been extensively investigated and it has been demonstrated that the binding of NGF to TrkA triggers TrkA autophosphorylation that, in turn, leads to a scaffolding role for TrkA and recruitment of several adapter proteins, such as Shc and phospholipase C, and enzymes that ultimately activate the ERK mitogen-activated protein kinases (MAPKs) and other downstream effectors [9,16,28,40]. In addition to its role in the TrkA/p75NTR high-affinity receptor complex, the p75NTR receptor

* Corresponding author. Fax: +1 412 692 6787.
E-mail address: nfschor@pitt.edu (N.F. Schor).

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appears to play independent roles in neural cell signaling. The binding of NGF to p75NTR has variably been found to induce or prevent apoptosis either in a ligand-independent or ligand-dependent fashion [10,21,29,41]. Efforts to identify p75NTR signaling pathways have similarly led to confusing and conflicting results [11,14,20,55]. As such, it has become imperative to identify the determinants of the effects and mechanisms of action of the p75NTR receptor. In the present studies, PC12 cells were used to investigate the effects and downstream effectors of the p75NTR. Our observations suggest that cholesterol biosynthesis plays a role in the pro-apoptotic effects of the p75NTR.

1. Materials and methods

1.1. Chemicals and reagents

Neocarzinostatin (NCS) has been described previously [57]. Briefly, NCS was obtained from Kayaku Pharmaceuticals Ltd (Tokyo, Japan), prepared as a 47 μM (0.5 mg/ml) stock solution in 0.015 M sodium acetate buffer with pH 5.0, stored in the dark at 4 °C for up to 2 weeks, and diluted with medium immediately before each experiment. NGF was obtained from Boehringer-Mannheim. The preparation and characterization of the monoclonal antibody mAbNGF30 have previously been described [47,57]. This antibody binds to the p75NTR binding site of NGF, blocking the binding of NGF to p75NTR and converting NGF to a TrkA-specific ligand. MC192 is a p75NTR-specific ligand [47]. All other antibodies, including a polyclonal anti-NGF antibody, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). 7-amino-actinomycin D (7-AAD; a fluorescent dye used to stain single-stranded DNA for the ascertainment of apoptosis using flow cytometry) and mevastatin were purchased from Sigma Chemical Corporation (St. Louis, MO). N-2 supplement (100×) was purchased from Invitrogen (Carlsbad, CA).

1.2. Cell cultures

As a population, native PC12 pheochromocytoma cells are p75NTR-positive (~40,000 p75NTR/cell and ~400 TrkA receptors/cell). p75NTR-negative (0 p75NTR/cell and ~400 TrkA receptors/cell) PC12 cells were selected and assayed for receptor content initially and again every several passages as we have previously described [52] and briefly summarize below.

Naturally arising subclones of PC12 cells were selected on the basis of absent cell surface p75NTR as determined by FACScan analysis using the monoclonal antibody mAbMC192, a ligand of the extracellular domain of p75NTR [2,7]. Subclones were then grown and examined by reverse transcriptase-polymerase chain reaction (RT-PCR) for mRNA and Western blotting for protein for p75NTR. The expression of TrkA was then determined in these cells and their native counterparts by functional (cell survival and differentiation) assay and by quantitative Western blotting with 203 serum directed against the TrkA intracellular domain. Studies were performed on pooled p75NTR-negative, TrkA-positive subclones of PC12 cells.

All cells used in these studies were demonstrated to be mycoplasma-free using a MycoTect Kit (GIBCO BRL Life Technologies). Both p75NTR-positive and p75NTR-negative PC12 cells were maintained with regular medium [Dulbecco's modified Eagle's medium supplemented with 10% horse serum, 5% fetal bovine serum (Atlanta Biologicals, Norcross, GA) and 1.1% penicillin/streptomycin (Invitrogen, Carlsbad, CA)] for at least 2 days, followed by replacement of regular medium with serum-free N-2-supplemented medium [Dulbecco's modified Eagle's medium supplemented with 1% 100× N-2 supplement solution (insulin: 500, human transferrin: 10,000, progesterone: 0.63, putrescine: 1611, selenite: 0.52 μg/ml)]. After 2 days of incubation with serum-free medium, the cells were treated and examined as indicated below. The cells were fed twice weekly.

1.3. Flow cytometric analysis of the apoptotic fraction of total cells

Apoptotic cells were quantified by flow cytometry as described previously [56]. The cells were harvested, washed once in PBS and once in PBS/0.05% saponin, and incubated with 7-AAD (4 μg/ml) for 30–60 min at room temperature in the dark. Then, DNA histograms were obtained using a CellQuest apparatus and CellQuest software (Becton Dickinson Biosciences, San Jose, CA). Data on 10^4 cells were collected. Electronic gates were set for viable and apoptotic cells with 2N–4N DNA and subnormal DNA contents, respectively, assuming 7-AAD staining intensity to be proportional to the DNA content [31]. Percent apoptosis was calculated as (number of apoptotic cells/number of total cells) × 100.

1.4. Western blotting detection of the phosphorylation of TrkA signaling pathway-related proteins

At the indicated time points after incubation with NGF (2 nM), p75NTR-positive and p75NTR-negative PC12 cells were lysed in RIPA buffer (10 mM Tris, pH 8, 150 mM NaCl, 0.1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 4 μg/ml aprotinin, 1 mM sodium orthovanadate). Subsequently, the protein concentrations of the lysates were estimated using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as a standard. An aliquot of each lysate containing 150 μg of protein was loaded onto each lane and electrophoresed on a 10% SDS-polyacrylamide gel, followed by blotting onto a nitrocellulose membrane (Bio-Rad Laboratories). After blotting, non-specific binding was blocked